Supporting Information

for

Antiferroptotic Activity of Phenothiazine Analogues: A Novel Therapeutic Strategy for Oxidative Stress Related Disease

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Experimental Procedures

All commercially available reagents were used without further purification. Anhydrous solvents were dried using routine protocols. All reactions were carried out under a nitrogen atmosphere in dry glassware with magnetic stirring. Column chromatography was conducted on 200-300 mesh silica gel. Analytical TLC was carried out by employing 0.25 mm silica gel plates (GF254) and visualization under UV light. NMR spectra were recorded on a Bruker 400 or Bruker 500 spectrometers. Chemical shifts are expressed in ppm, and *J* values are given in Hz. All compounds evaluated for their biological effects were > 95% pure. Chemicals employed for the bioassays, including ferrostatin-1 (Fer-1), liproxstatin-1 (Lip-1), RSL3 ((1*S*,3*R*)-RSL3), erastin, α-tocopherol, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and galactose, were purchased from Sigma Aldrich. Fluorescent probes, including C_{11}-BODIPY$^{581/591}$(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid), and The Live/Dead® Viability/Cytotoxicity Kit were obtained from Invitrogen (Thermo Fisher Scientific). Luciferase-linked ATPase enzymatic assay (ViaLight Plus proliferation/cytoxicity kit was purchased from Lonza (Walkersville, MD). FRDA lymphocytes (catalogue number GM15850,) and FRDA fibroblasts cells (catalogue number GM04078,) were obtained from Coriell Cell Repositories (Camden, NJ, USA). Lymphocytes were cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY) with 15% fetal calf serum, 2 mM glutamine (HyClone, South Logan, UT) and 1% penicillin–streptomycin antibiotic supplement (Cellgro, Manassas, VA). Cells were passaged daily to maintain them in log phase growth and kept at a nominal concentration of 5-10 $\times 10^5$ mL. FRDA fibroblasts were cultured in 64% (v/v) Eagle’s minimal essential medium (MEM), lacking phenol red with Eagle’s balanced salt (EBS) and 25% M199 with EBS (Gibco) supplemented with 10% (v/v) fetal bovine serum albumin (HyClone), 1% penicillin–streptomycin antibiotic mix, 10 µg/mL insulin (Gibco), 10 ng/mL endothelial growth factor (EGF), 10 ng/mL basic fibroblast growth factor (Gibco) and 2 mM glutamine. Cells were grown in 75 cm^2 culture flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were fed twice weekly and split every third day at a ratio of 1:3. All experiments were conducted with FRDA cells from passages 8-14.

Synthesis of MB/MV Analogues

Heptyltriphenylphosphonium Bromide (6). To a stirred solution of 1.00 g (3.81 mmol) of triphenylphosphine in 30 mL of anhydrous toluene was added 598 μL (3.81 mmol) of 1-bromoheptane at room temperature. The reaction mixture was stirred at reflux overnight. The cooled reaction mixture was filtered, and the collected white precipitate was washed with an additional 20 mL of toluene and dried to afford **6** as a white solid; yield 1.51 g (90%); silica gel TLC R_f 0.3 (10:1 CH_2Cl_2-MeOH). This material was used directly without further purification.

Octyltriphenylphosphonium Bromide (7). To a stirred solution containing 1.00 g (3.81 mmol) of triphenylphosphine in 30 mL of anhydrous toluene was added 658 μL (3.81 mmol) of 1-bromooctane at room temperature. The reaction mixture was stirred at reflux overnight. The cooled reaction mixture was filtered, and the collected white precipitate was washed with an additional 20 mL of toluene and dried to afford **7** as a white solid; yield 1.49 g (86%); silica gel TLC R_f 0.4 (10:1 CH_2Cl_2-MeOH). This material was used directly without further purification.

Nonyltriphenylphosphonium Bromide (8). To a stirred solution containing 1.00 g (3.81 mmol) of triphenylphosphine in 30 mL of anhydrous toluene was added 728 µL (3.81 mmol) of 1-bromononane at room temperature. The reaction mixture was stirred at reflux overnight. The cooled reaction mixture was filtered, and the collected white precipitate was washed with an additional 20 mL of toluene and dried to afford **8** as a white solid; yield 1.46 g (82 %); silica gel TLC R_f 0.4 (10:1 CH_2Cl_2-MeOH). This material was used directly without further purification.

Triphenyl(undecyl)phosphonium Bromide (9). To a stirred solution containing 1.00 g (3.81 mmol) of triphenylphosphine in 30 mL of anhydrous toluene was added 849 µL (3.81 mmol) of 1-bromoundecane at room temperature. The reaction mixture was stirred at reflux overnight. The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (10 × 5 cm). Elution with 20:1 CH_2Cl_2-MeOH afforded **9** as a colorless oil: yield 1.61 g (85 %); silica gel TLC R_f 0.5 (10:1 CH_2Cl_2-MeOH).

***tert*-Butyl 2-Cyano-10*H*-phenothiazine-10-carboxylate (10).** To a solution of 2.00 g, (8.90 mmol) of 2-cyanophenothiazine in in 25 mL of anhydrous DMF was added 536 mg (13.4 mmol, 60% in mineral oil) of $NaBH_4$ in an ice bath and the reaction mixture was stirred for an additional 30 min. A sample of 2.30 g (10.7 mmol) of di-*tert*-butyl-dicarbonate was then added. The reaction mixture was stirred at room temperature overnight, after which silica gel TLC analysis indicated the consumption of the starting material. The reaction mixture was quenched with 25 mL of saturated NH_4Cl, and then extracted with two 30-mL portions of CH_2Cl_2. The combined organic extract was washed with 20 mL of brine, dried over $MgSO_4$ and concentrated under diminished pressure to give the crude product. Purification by flash chromatography on a silica gel column (15 × 5 cm) afforded pure **10** as a white solid: yield 2.20 g (75%); silica gel TLC R_f 0.4 (3:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.31 (s, 9H), 7.01 (td, 1H, J = 7.6, 1.3 Hz), 7.09-7.16 (m, 2H), 7.22 (d, 2H, J = 1.0 Hz), 7.31 (dd, 1H, J = 8.0, 0.9 Hz) and 7.62 (t, 1H, J = 1.0 Hz); ^{13}C NMR ($CDCl_3$) δ 28.1, 83.2, 110.2, 118.2, 126.7, 127.3, 127.4, 127.5, 128.1, 129.2, 130.4, 130.7, 137.7, 139.0, 139.1 and 151.9.

***tert*-Butyl 2-Formyl-10*H*-phenothiazine-10-carboxylate (11).** To a stirred solution of 1.00 g (3.10 mmol) of **10** (1.0 g, 3.1 mmol) in 30 mL of anhydrous CH_2Cl_2 at –78 °C under a nitrogen atmosphere, was added dropwise 4.6 mL of a 1M solution in toluene (4.6 mmol) of DiBAl-H and the resulting reaction mixture was stirred at –78 °C for 3 h. Silica gel TLC analysis indicated the consumption of starting material, and the reaction mixture was diluted with CH_2Cl_2, washed successively with H_2O and brine, then dried over $MgSO_4$, filtered, and concentrated under diminished pressure to afford the crude product. The crude residue was purified by flash chromatography (10 × 5 cm column), elution with 10:1 hexane-EtOAc, to give pure **11** as a yellow solid: yield 850 mg (85%); silica gel TLC R_f 0.5 (3:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H), 7.12 (td, 1H, J = 7.6, 1.3 Hz), 7.25 (ddd, 2H, J = 15.6, 7.9, 1.5 Hz), 7.40 (d, 1H, J = 8.0 Hz), 7.46 (dd, 1H, J = 8.1, 1.2 Hz), 7.60 (dd, 1H, J = 8.0, 1.7 Hz), 7.95 (d, 1H, J = 1.6 Hz) and 9.91 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 28.1, 82.8, 126.5, 126.6, 127.1, 127.3, 127.5, 127.9, 128.4, 130.5, 135.1, 138.0, 139.1, 140.3, 152.1 and 190.9.

***tert*-Butyl (*E*)-2-(Oct-1-en-1-yl)-10*H*-phenothiazine-10-carboxylate (12).** To a stirred solution of 99.0 mg (0.23 mmol) of (1-heptyl)triphenylphosphonium bromide in 5 mL of anhydrous THF at –78 °C under a nitrogen atmosphere and kjept for 15 min, was added dropwise 0.3 mL of a 1

M THF solution (0.3 mmol) of NaHMDS. The resulting reaction mixture was stirred in an ice bath for an additional 3 h. The reaction was cooled to –78 °C, and 50.0 mg (0.15 mmol) of **11** dissolved in 2 mL of anhydrous THF was added. Stirring was continued for a further 18 h at 0 °C. Silica gel TLC analysis indicated the consumption of starting material, and the reaction was quenched with saturated NH_4Cl and extracted with two 20-mL portions of CH_2Cl_2. The combined extract was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure to give the crude product. The residue purified by chromatography on a silica gel column (10 × 2 cm), elution with 150:1 hexane-EtOAc, to afford **12** as a white solid: yield 51.3 mg (82%); silica gel TLC R_f 0.5 (20:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88-1.03 (m, 3H), 1.30-1.65 (m, 17H), 2.35-2.55 (m, 2H), 5.65-5.83 (m, 1H), 6.43 (d, 1H, J = 11.2 Hz), 7.07-7.20 (m, 2H), 7.22-7.41 (m, 3H) and 7.52-7.68 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.7, 28.2, 28.8, 29.1, 30.0, 31.8, 81.9, 126.1, 126.5, 126.6, 127.0, 127.3, 127.4, 127.5, 128.0, 130.0, 132.2, 133.8, 136.6, 138.6, 138.8 and 152.4; mass spectrum (ESI) m/z 410.2 $[M + H]^+$ ($C_{25}H_{32}NO_2S$ requires 410.2).

***tert*-Butyl (*E*)-2-(Non-1-en-1-yl)-10*H*-phenothiazine-10-carboxylate (13).** To a stirred solution containing 105 mg (0.23 mmol) of (1-octyl)triphenylphosphonium bromide in 5 mL of anhydrous THF cooled under argon at –78 °C and kept for 15 min, was added dropwise 0.30 mL (0.30 mmol) of a 1 M solution of NaHMDS in THF. The resulting reaction mixture was stirred in an ice bath for an additional 3 h. After this time, the reaction mixture was cooled to –78 °C, and a solution containing 50.0 mg (0.15 mmol) of **11** in 2 mL of anhydrous THF was added dropwise. Stirring was continued for a further 18 h at 0 °C. The reaction was quenched by adding 5 mL of saturated NH_4Cl, and extracted with two 20-mL portions of CH_2Cl_2. The combined organic phase was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm), elution with 150:1 hexane-EtOAc to afford **13** as a white solid: yield 51.0 mg (80%); silica gel TLC R_f 0.5 (20:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H, J = 6.8 Hz), 1.34-1.55 (m, 10H), 1.57 (s, 9H), 2.42 (q, 2H, J = 7.2 Hz), 5.75 (dt, 1H, J = 11.7, 7.3 Hz), 6.45 (d, 1H, J = 11.6 Hz), 7.12-7.20 (m, 2H), 7.27-7.35 (m, 2H), 7.38 (d, 1H, J = 7.7 Hz), 7.55 (s, 1H) and 7.61 (d, 1H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.7, 28.2, 28.7, 29.3, 29.4, 30.0, 31.9, 81.9, 126.1, 126.5, 126.6, 127.0, 127.3, 127.4, 127.5, 127.9, 130.0, 132.3, 133.9, 136.7, 138.6, 138.8 and 152.5; mass spectrum (ESI) m/z 424.2 $(M+H)^+$ ($C_{26}H_{34}NO_2S$ requires 424.2).

***tert*-Butyl (*E*)-2-(Dec-1-en-1-yl)-10*H*-phenothiazine-10-carboxylate (14).** To a stirred solution containing 108 mg (0.23 mmol) of (1-nonyl)triphenylphosphonium bromide in 5 mL of anhydrous THF cooled under argon at –78 °C and kept for 15 min, was added dropwise 0.30 mL (0.30 mmol) of a 1 M solution of NaHMDS in THF. The resulting reaction mixture was stirred in an ice bath for an additional 3 h. After this time, the reaction mixture was cooled to –78 °C, and a solution containing 50.0 mg (0.15 mmol) of **11** in 2 mL of anhydrous THF was added dropwise. Stirring was continued for a further 18 h at 0 °C. The reaction was quenched by adding 5 mL of saturated NH_4Cl, and extracted with two 20-mL portions of CH_2Cl_2. The combined organic phase was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm), elution with 150:1 hexane-EtOAc to afford **14** as a white solid: yield 51.1 mg (78%); silica gel TLC R_f 0.5 (20:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, J = 5 Hz, 3H), 1.25-1.53 (m, 12H), 1.57 (s, 9H), 2.41 (q, 2H, J = 6.6 Hz), 5.72-5.76 (m, 1H), 6.43 (d, 1H, J

= 11.6 Hz), 7.13-7.19 (m, 2H), 7.22-7.30 (m, 2H), 7.37 (d, 1H, *J* = 10 Hz), 7.54 (s, 1H) and 7.60 (d, 1H, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.8, 28.3, 28.8, 29.4, 29.5, 29.6, 30.1, 32.0, 82.0, 126.1, 126.5, 126.7, 127.0, 127.4, 127.5, 127.6, 127.9, 130.1, 132.3, 133.9, 136.7, 138.6, 138.9 and 152.5; mass spectrum (ESI) *m/z* 438.2 $(M+H)^+$ ($C_{27}H_{36}NO_2S$ requires 438.2).

***tert*-Butyl (*E*)-2-(Dodec-1-en-1-yl)-10*H*-phenothiazine-10-carboxylate (15).** To a stirred solution containing 114 mg (0.23 mmol) of (1-undecyl)triphenylphosphonium bromide in 5 mL of anhydrous THF cooled under argon at –78 °C and kept for 15 min, was added dropwise 0.30 mL (0.30 mmol) of a 1 M solution of NaHMDS in THF. The resulting reaction mixture was stirred in an ice bath for an additional 3 h. After this time, the reaction mixture was cooled to –78 °C, and a solution containing 50.0 mg (0.15 mmol) of **11** in 2 mL of anhydrous THF was added dropwise. Stirring was continued for a further 18 h at 0 °C. The reaction was quenched by adding 5 mL of saturated NH_4Cl, and extracted with two 20-mL portions of CH_2Cl_2. The combined organic phase was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm), elution with 150:1 hexane-EtOAc to afford **15** as a white solid: yield 52.4 mg (75%); silica gel TLC R_f 0.5 (20:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 6.7 Hz,), 1.25-1.35 (m, 16H), 1.49 (s, 9H), 2.32 (q, 2H, *J* = 7.3 Hz), 5.67 (dt, 1H, *J* = 11.6, 7.3 Hz), 6.36 (d, *J* = 11.6 Hz, 1H), 7.08 (d, *J* = 8.0 Hz, 1H), 7.13-7.19 (m, 1H), 7.22-7.30 (m, 2H), 7.34 (dd, 1H, *J* = 7.6, 4.3 Hz), 7.43 (s, 1H) and 7.53 (d, 1H, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 28.2, 28.7, 29.4, 29.5, 29.6, 29.6, 29.6, 29.9, 31.9, 82.0, 126.0, 126.5, 126.6, 126.9, 127.3, 127.4, 127.5, 127.7, 129.9, 132.2, 133.9, 136.7, 138.5, 138.8 and 152.5; mass spectrum (ESI) *m/z* 466.2 $(M+H)^+$ ($C_{29}H_{40}NO_2S$ requires 466.2).

***tert*-Butyl 2-Octyl-10*H*-phenothiazine-10-carboxylate (16).** To 30 mL of 7:3 EtOH-CH_2Cl_2 was added 500 mg (1.20 mmol) of **12**. The solution was purged with nitrogen for 20 min, and then treated with 125 mg of 10% palladium-on-carbon. The suspension was stirred under an atmosphere of H_2 (40 psi) for 3 h. The reaction mixture was filtered through a Celite pad and the filtrate was concentrated under diminished pressure to give crude product **16** in quantitative yield. The crude product was used directly without further purification.

***tert*-Butyl 2-Nonyl-10*H*-phenothiazine-10-carboxylate (17)**. A solution containing 508 mg (1.20 mmol) of **13** in 30 mL of 7:3 EtOH-CH_2Cl_2 was purged with argon for 20 min. To the resulting solution was added 125 mg of 10% palladium-on-carbon. The suspension was stirred at room temperature under a hydrogen atmosphere (40 psi) for 3 h. The reaction mixture was filtered through a Celite pad and the filtrate was concentrated under diminished pressure. The crude product (**17**) was obtained in quantitative yield and was used for the next reaction without further purification.

***tert*-Butyl 2-Decyl-10*H*-phenothiazine-10-carboxylate (18).** A solution containing 524 mg (1.20 mmol) of **14** in 30 mL of 7:3 EtOH-CH_2Cl_2 was purged with argon for 20 min. To the resulting solution was added 125 mg of 10% palladium-on-carbon. The suspension was stirred at room temperature under a hydrogen atmosphere (40 psi) for 3 h. The reaction mixture was filtered through a Celite pad and the filtrate was concentrated under diminished pressure. The crude product (**18**) was obtained in quantitative yield and was used for the next reaction without further purification.

***tert*-Butyl 2-Dodecyl-10*H*-phenothiazine-10-carboxylate (19).** A solution containing 558 mg (1.20 mmol) of **15** in 30 mL of 7:3 EtOH-CH_2Cl_2 was purged with argon for 20 min. To the resulting solution was added 125 mg of 10% palladium-on-carbon. The suspension was stirred at room temperature under a hydrogen atmosphere (40 psi) for 3 h. The reaction mixture was filtered through a Celite pad and the filtrate was concentrated under diminished pressure. The crude product (**19**) was obtained in quantitative yield and was used for the next reaction without further purification.

2-Octyl-10*H*-phenothiazine (20). To a stirred solution of 200 mg (0.50 mmol) of **16** in 15 mL of anhydrous CH_2Cl_2 was added dropwise 300 μL (4.00 mmol) of trifluoroacetic acid at room temperature under nitrogen. The reaction mixture was stirred for 4 h, then neutralized with saturated $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. Crude **20** was utilized in the next step without further purification.

2-Nonyl-10*H*-phenothiazine (21). To a stirred solution containing 212 mg (0.50 mmol) of **17** in 15 mL of anhydrous CH_2Cl_2 was added dropwise 300 μL (4.00 mmol) of trifluoroacetic acid at room temperature under nitrogen protection. The reaction mixture was stirred for 4 h. Then, the reaction was neutralized with 5 mL of saturated $NaHCO_3$ and extracted with 20 mL of CH_2Cl_2. The organic phase was washed with brine, dried with $MgSO_4$, and concentrated under diminished pressure. The crude product (**21)** was used for the next step without further purification.

2-Decyl-10*H*-phenothiazine (22). To a stirred solution containing 219 mg (0.50 mmol) of **18** in 15 mL of anhydrous CH_2Cl_2 was added dropwise 300 μL (4.00 mmol) of trifluoroacetic acid at room temperature under nitrogen protection. The reaction mixture was stirred for 4 h. Then, the reaction was neutralized with 5 mL of saturated $NaHCO_3$ and extracted with 20 mL of CH_2Cl_2. The organic phase was washed with brine, dried with $MgSO_4$, and concentrated under diminished pressure. The crude product (**22)** was used for the next step without further purification.

2-Dodecyl-10*H*-phenothiazine (23). To a stirred solution containing 233 mg (0.50 mmol) of **19** in 15 mL of anhydrous CH_2Cl_2 was added dropwise 300 μL (4.00 mmol) of trifluoroacetic acid at room temperature under nitrogen protection. The reaction mixture was stirred for 4 h. Then, the reaction was neutralized with 5 mL of saturated $NaHCO_3$ and extracted with 20 mL of CH_2Cl_2. The organic phase was washed with brine, dried with $MgSO_4$, and concentrated under diminished pressure. The crude product (**23)** was used for the next step without further purification.

***N*-(7-(Dimethylamino)-3*H*-phenothiazin-3-ylidene-2-octyl)-*N*-methylmethanaminium Iodide (1a).** To a solution containing 155 mg (0.50 mmol) of **20** in 15 mL of anhydrous CH_2Cl_2 was added 380 mg (1.50 mmol) of iodine and the reaction mixture was stirred in dark for 30 min. A solution containing 1.0 mL (2.0 mmol) of 2 M dimethylamine in THF was added dropwise, and the resulting reaction mixture was stirred at room temperature for another 4 h. The reaction mixture was concentrated under diminished pressure to give the crude product, which was purified by flash chromatography on a silica gel column (15 × 5 cm). Elution with 30:1 CH_2Cl_2-MeOH afforded **1a** as a blue solid: yield 89 mg (35% for two steps); silica gel TLC R_f 0.4 (10:1 CH_2Cl_2-MeOH); ^1H NMR ($CDCl_3$) δ 0.80-0.83 (m, 3H), 1.20-1.37 (m, 10H), 1.57-1.71 (m, 2H), 2.75-2.87 (m, 2H), 3.34 (s, 6H), 3.49 (s, 6H), 7.28-7.44 (m, 2H), 7.65-7.98 (m, 2H) and 8.61 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 29.1, 29.4, 29.5, 30.1, 31.8, 35.0, 42.6, 44.7, 107.1, 111.2, 120.0, 131.7, 135.5, 137.0, 137.2, 137.3, 138.5, 138.9, 154.0 and 158.0; mass spectrum (ESI) *m/z* 396.2452 $[M-I]^+$ ($C_{24}H_{34}N_3S$ requires 396.2468).

***N*-(7-(Dimethylamino)-3*H*-phenothiazin-3-ylidene-2-nonyl)-*N*-methylmethanaminium Iodide (2a).** To a stirred solution containing 162 mg (0.50 mmol) of **21** in 15 mL of anhydrous CH_2Cl_2 was added 380 mg (1.50 mmol) of iodine and the reaction mixture was stirred for 30 min in the dark. A solution containing 1.00 mL (2.00 mmol) of 2 M dimethylamine in THF was added dropwise, and the resulting mixture was stirred at room temperature for another 4 h. The reaction mixture was concentrated under diminished pressure to give the crude product. The residue was purified by flash chromatography on a silica gel column (15 × 5 cm). Elution with 30:1 CH_2Cl_2-MeOH afforded **2a** as a blue solid: yield 67.1 mg (25% for two steps); silica gel TLC R_f 0.4 (10:1 CH_2Cl_2-MeOH); ^1H NMR ($CDCl_3$) δ 0.74 (t, 3H, J = 6.1 Hz), 1.14-1.29 (m, 12H), 1.53-1.62 (m, 2H), 2.71-2.80 (m, 2H), 3.28 (s, 6H), 3.40 (s, 6H), 7.12-7.24 (m, 2H), 7.60-7.79 (m, 2H) and 8.61 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 29.2, 29.3, 29.4, 30.1, 31.7, 34.0, 34.9, 42.8, 44.8, 107.0, 111.1, 120.1, 131.4, 135.1, 136.7, 137.0, 137.1, 138.3, 138.7, 153.9 and 157.7; mass spectrum (ESI) *m/z* 410.2607 $[M-I]^+$ ($C_{25}H_{36}N_3S$ requires 410.2624).

***N*-(7-(Dimethylamino)-3*H*-phenothiazin-3-ylidene-2-decyl)-*N*-methylmethanaminium Iodide (3a).** To a stirred solution containing 169 mg (0.50 mmol) of **22** in 15 mL of anhydrous CH_2Cl_2 was added 380 mg (1.50 mmol) of iodine and the reaction mixture was stirred for 30 min in the dark. A solution containing 1.00 mL (2.00 mmol) of 2 M dimethylamine in THF was added dropwise, and the resulting mixture was stirred at room temperature for another 4 h. The reaction mixture was concentrated under diminished pressure to give the crude product. The residue was purified by flash chromatography on a silica gel column (15 × 5 cm). Elution with 30:1 CH_2Cl_2-MeOH afforded **3a** as a blue solid: yield 55.1 mg (20% for two steps); silica gel TLC R_f 0.4 (10:1 CH_2Cl_2-MeOH); ^1H NMR ($CDCl_3$) δ 0.67 (t, 3H, J = 5.6 Hz), 1.05-1.25 (m, 14H), 1.46-1.55 (m, 2H), 2.62-2.73 (m, 2H), 3.20 (s, 6H), 3.33 (s, 6H), 7.11-7.25 (m, 2H), 7.51-7.67 (m, 2H) and 8.52 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.5, 29.1, 29.3, 29.4, 30.1, 31.7, 33.9, 35.0, 35.1, 42.8, 44.8, 106.9, 111.0, 120.1, 131.3, 135.0, 136.6, 136.9, 137.0, 138.3, 138.7, 153.8 and 157.6; mass spectrum (ESI) *m/z* 424.2790 $[M - I]^+$ ($C_{26}H_{38}N_3S$ requires 424.2781).

***N*-(7-(Dimethylamino)-3*H*-phenothiazin-3-ylidene-2-dodecyl)-*N*-methylmethanaminium Iodide (4a).** To a stirred solution containing 183 mg (0.50 mmol) of **23** in 15 mL of anhydrous CH_2Cl_2 was added 380 mg (1.50 mmol) of iodine and the reaction mixture was stirred for 30 min in the dark. A solution containing 1.00 mL (2.00 mmol) of 2 M dimethylamine in THF was added dropwise, and the resulting mixture was stirred at room temperature for another 4 h. The reaction mixture was concentrated under diminished pressure to give the crude product. The residue was purified by flash chromatography on a silica gel column (15 × 5 cm). Elution with 30:1 CH_2Cl_2-MeOH afforded **4a** as a blue solid: yield 66.6 mg (23% for two steps); silica gel TLC R_f 0.4 (10:1 CH_2Cl_2-MeOH); ^1H NMR (MeOD) δ 0.89 (t, 3H, J = 6.7 Hz), 1.24-1.48 (m, 18H), 1.66-1.79 (m, 2H), 2.88-2.98 (m, 2H), 3.29 (s, 6H), 3.44 (s, 6H), 7.42-7.51 (m, 2H), 7.60

(dd, 1H, J = 9.7, 1.9 Hz), 7.93 (s, 1H) and 8.01 (d, 1H, J = 9.6 Hz); ^{13}C NMR (MeOD) δ 13.0, 22.3, 28.9, 29.0, 29.0, 29.2, 29.3, 29.3, 29.4, 29.5, 31.7, 33.6, 40.3, 42.9, 106.2, 110.5, 120.0, 131.3, 135.7, 136.4, 136.5, 137.3, 138.3, 138.4, 154.7 and 158.2; mass spectrum (ESI) *m/z* 452.3099 $[M - I]^+$ ($C_{28}H_{42}N_3S$ requires 452.3094).

7-(Dimethylamino)-2-octyl-3*H*-phenothiazin-3-one (1b). To a stirred solution containing 100 mg (0.19 mmol) of **1a** in 7 mL of 6:1 THF-H_2O was added 34.0 mg (0.60 mmol) of potassium hydroxide. The reaction mixture was stirred at room temperature for 30 min at which time the color of the solution had changed from blue to red. The reaction mixture was quenched with water and extracted with two 20-mL portions of CH_2Cl_2. The combined organic extract was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure to give the crude product. The crude product was purified by flash chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 hexane-EtOAc afforded **1b** as a red solid: yield 14 mg (20%); silica gel TLC R_f 0.4 (3:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3H, J = 6.9 Hz), 1.26-1.40 (m, 10H), 1.69-1.73 (m, 2H), 2.-2.73 (m, 2H), 2.90 (s, 6H), 6.75 (d, 1H, J = 2.1 Hz), 6.91 (dd, 1H, J = 9.8, 2.1 Hz), 6.94 (s, 1H), 7.60 (d, 1H. J = 9.8 Hz) and 7.72 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 29.2, 29.4, 29.7, 30.0, 31.5, 31.9, 43.9, 112.7, 119.0, 123.3, 133.7, 134.5, 135.3, 135.4, 136.3, 139.6, 143.1, 155.7 and 182.1; mass spectrum (ESI) *m/z* 369.2002 $[M + H]^+$ $C_{22}H_{29}N_2OS$ requires 369.1995).

7-(Dimethylamino)-2-nonyl-3*H*-phenothiazin-3-one (2b). To a stirred solution containing 107 mg (0.20 mmol) of **2a** in 7 mL of 6:1 THF-H_2O was added 34.0 mg (0.60 mmol) of potassium hydroxide. The reaction mixture was stirred at room temperature for 30 min at which time the color of the solution had changed from blue to red. The reaction mixture was quenched with water and extracted with two 20-mL portions of CH_2Cl_2. The combined organic extract was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 hexane-EtOAc afforded **2b** as a red solid: yield 20.0 mg (26%); silica gel TLC R_f 0.4 (3:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.9 Hz), 1.26-1.44 (m, 12H), 1.66-1.76 (m, 2H), 2.70 (t, 2H, J = 7.5 Hz), 2.88 (s, 6H), 6.71 (d, 1H, J = 2.1 Hz), 6.88 (dd, 1H, J = 9.8, 2.1 Hz), 6.91 (s, 1H), 7.58 (d, 1H, J = 9.8 Hz) and 7.69 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 29.3, 29.5, 29.6, 29.7, 29.9, 31.5, 31.9, 43.9, 112.6, 119.1, 123.2, 133.8, 134.4, 135.3, 135.4, 136.2, 139.5, 143.1, 155.7 and 182.2; mass spectrum (ESI) *m/z* 383.2159 $[M + H]^+$ ($C_{23}H_{31}N_2OS$ requires 383.2152).

2-Decyl-7-(dimethylamino)-3*H*-phenothiazin-3-one (3b). To a stirred solution containing 110 mg (0.20 mmol) of **3a** in 7 mL of 6:1 THF-H_2O was added 34.0 mg (0.60 mmol) of potassium hydroxide. The reaction mixture was stirred at room temperature for 30 min at which time the color of solution had changed from blue to red. The reaction mixture was quenched with water and extracted with two 20-mL portions of CH_2Cl_2. The combined organic phase was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 hexane-EtOAc afforded **3b** as a red solid: yield 23.0 mg (29%); silica gel TLC R_f 0.5 (3:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3H, J = 6.9 Hz), 1.24-1.43 (m, 14H), 1.66-1.76 (m, 2H), 2.72 (t, 2H, J = 7.5 Hz), 2.90 (s, 6H), 6.75 (s, 1H), 6.89 (dd, 1H, J = 9.8, 2.2 Hz), 6.94 (s, 1H), 7.60 (d, 1H, J = 9.8 Hz) and 7.71 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.8, 29.4, 29.6, 29.7, 29.7, 29.8, 30.1, 31.6, 32.0, 43.9, 112.7, 119.0, 123.3, 133.7, 134.5, 135.3, 135.4, 136.3, 139.6, 143.1, 155.7 and 182.1; mass spectrum (ESI) m/z 397.2310 $[M + H]^+$ ($C_{24}H_{33}N_2OS$ requires 397.2308).

7-(Dimethylamino)-2-dodecyl-3*H*-phenothiazin-3-one (4b). To a stirred solution containing 116 mg (0.20 mmol) of **4a** in 7 mL of 6:1 THF-H_2O was added 34.0 mg (0.60 mmol) of potassium hydroxide. The reaction mixture was stirred at room temperature for 30 min at which time the color of solution had changed from blue to red. The reaction mixture was quenched with water and extracted with two 20-mL portions of CH_2Cl_2. The combined organic phase was washed with brine, dried over $MgSO_4$, and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 hexane-EtOAc afforded **4b** as a red solid: yield 28.8 mg (34%); silica gel TLC R_f 0.5 (3:1 hexane-EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.9 Hz), 1.25-1.43 (m, 18H), 1.70 (dt, 2H, J = 15.6, 7.7 Hz), 2.67-2.75 (m, 2H), 2.88 (s, 6H), 6.71 (d, 1H, J = 2.2 Hz), 6.88 (dd, 1H, J = 9.8, 2.2 Hz), 6.91 (s, 1H), 7.57 (d, 1H, J = 9.8 Hz) and 7.69 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 29.4, 29.4, 29.5, 29.6, 29.7, 29.7, 29.7, 30.0, 31.5, 31.9, 43.9, 112.7, 119.1, 123.2, 133.8, 134.4, 135.3, 135.3, 136.3, 139.6, 143.1, 155.7 and 182.3; mass spectrum (ESI-MS) m/z 425.2627 $[M + H]^+$ ($C_{26}H_{37}N_2OS$ requires 425.2621).

Bioassay Protocols

Assessment of Lipid Peroxidation. Lipid peroxidation in live cells can be visualized using the oxidation-sensitive fluorescent probe C_{11}- BODIPY$^{581/591}$. Upon oxidation of this probe, the fluorescence properties shift from a red-emitting form (595 nm) to a green emitting form (520 nm). Changes in fluorescence indirectly reflect the oxidation of unsaturated fatty acids. A quantitative analysis of lipid peroxidation in FRDA lymphocytes, challenged with 2 µM RSL3 in the presence or absence of the test compounds for 2 h was obtained by FACS analysis using the specific dye C_{11}- BODIPY$^{581/591}$. Briefly, FRDA lymphocytes (5 x 10^5 cell/mL) were treated with the test compounds (dissolved in DMSO and added to the assay medium at final DMSO concentrations < 0.5%) at varying concentrations and incubated at 37 °C overnight in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 2 µM of RSL3 and then incubated for 90 minutes in a humidified atmosphere containing 5% CO_2 in air at 37 °C. Cells were treated with 2 µM of C_{11}- BODIPY$^{581/591}$ in phenol red-free RPMI-1640 media and incubated at 37 °C in the dark for 30 min. Treated cells were collected by centrifugation at 300 × g for 3 min and then washed with phosphate buffered saline (PBS). Cells were resuspended in 300 µL of PBS and were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), to monitor the change in intensity of the C_{11}- BODIPY$^{581/591}$-green (oxidized) fluorescence signal using 488 nm excitation laser and FL1-H channel 538 nm emission filter. In each analysis, 10,000 events were recorded. The experiments shown are representative of at least two independent experiments. Final data output was a calculated EC_{50} value determined graphically from the dose–response curves using the nonlinear regression curve fit analysis model.

Erastin Cell Survival Assay (FRDA fibroblasts). The ability of the test compounds to confer cytoprotection to ferroptosis inducer erastin in FRDA fibroblasts was determined by assessing the depletion of cellular ATP using a commercially available luciferase-linked ATPase enzymatic assay (ViaLight® Plus proliferation/cytotoxicity Kit (Lonza, Walkersville, MD) according to the directions of the manufacturer. Briefly, FRDA fibroblasts (GM04078) were plated in 96-well microtiter plates at a density of 3000 cell/well (50 µL) (Costar, Corning, NY). The plates were incubated at 37 °C for 24 h in an atmosphere having 95% humidity and 5% CO_2 to allow attachment of the cells to the culture plates and to allow the cell density reach 60-70% confluency. The next day the test compounds were dissolved in DMSO, diluted to the appropriate concentrations in fresh cell culture media and the cells were then treated with the test compounds (final DMSO concentration was < 0.5%). Twelve hours later, 50 µL of erastin solution in culture medium were added to the wells, resulting in 4 µM final concentration. Cell viability in each well was determined 48 h after erastin addition by measuring the intracellular ATP content using the ViaLight® -Plus ATP monitoring reagent kit (Lonza, Walkersville, MD) according to the manufacturer's protocol. The ATP concentration was determined by reading luminescence using a luminator (Clarity™ luminescence microplate reader). The percent of viable cells relative to non-treated control was determined for each well. The experiments shown are representative of at least two independent experiments. Final data output was a calculated EC_{50} value determined graphically from the dose–response curves using the nonlinear regression curve fit analysis model.

Measurement of Cellular ATP Concentration. The total cellular ATP levels in FRDA lymphocytes were measured using a commercially available luciferase-linked ATPase enzymatic assay (ViaLight Plus proliferation/cytotoxicity Kit (Lonza, Walkersville, MD) according to the manufacturer's directions. Briefly, lymphocytes (2×10^5 cell/mL) were plated (1 mL) in 24-well plates, treated with the test compounds (dissolved in DMSO and added to the assay medium at final DMSO concentrations <0.5%) at final compound concentrations of 0.1, 0.25, 0.5 and 2.5 µM, and then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2 in air. Cells in each well were mixed and transferred (100 µL) to 96-well microtiter white-walled cell culture plates (Costar, Corning, NY). The total intracellular ATP level was measured in a luminator (Clarity™ luminescence microplate reader) using an ATP Bioluminescence Assay Kit (ViaLight®-Plus ATP monitoring reagent kit, Lonza) following the manufacturer's protocol. The total ATP level was expressed as a percentage of untreated control. Data are reported as the mean of at least three independent experiments.

Mitochondrial Membrane Potential ($\Delta\psi_m$) Assay. The lipophilic dye cationic dye, JC-1, was used to evaluate the mitochondrial potential. JC-1 displays membrane potential-dependent accumulation in the mitochondria and has dual-fluorescent properties being red as aggregates and green as monomers. This probe provides a simple, fluorescence-based method for distinguishing between healthy and apoptotic cells. Changes in $\Delta\psi_m$ were assessed by flow cytometry as described before. Briefly, FRDA lymphocytes cells (5×10^5 cells) were pretreated with or without the test compounds for 16 h. The cells were incubated at 37 °C in the dark for 20 min with 1 µM JC-1. Cells were collected by centrifugation at 300 × g for 3 min and washed with PBS. The cells were resuspended in PBS supplemented with 20 mM glucose and were analyzed immediately by flow cytometry using a 488 nm excitation laser and the FL1-H channel 530 ±15 nm emission filter and the FL2-H channel 585 ±15 nm emission filter. For each analysis, 10,000 events were recorded and analyzed using C6 Accuri software. FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone), a mitochondrial uncoupler, was used to produce a negative control. Results expressed as the percentage of cells with intact $\Delta\psi_m$ were verified by running duplicates and repeating each experiment as a second independent experiment.

Cytotoxicity Assay. MB/MV analogues were tested for their cytotoxicity in FRDA lymphocytes using simultaneous staining with a two-color fluorescence assay, the Live/ Dead® Viability/Cytotoxicity Kit (Molecular Probes). Briefly, one mL of FRDA lymphocyte cells (5×10^5 cells) were plated in a 24-well plate in glucose free media (galactose 25 mM), treated with the test compounds and incubated at 37 °C for 24 h or 48 h in a humidified atmosphere containing 5% CO_2 in air. Cells were collected by centrifugation at 300 × g for 3 min and washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline containing 25 mM galactose. The cell suspension was stained with 0.1 µM calcein AM and 0.2 µM EthD-1 and incubated in the dark at 37 °C for 15 min. Cells were collected by centrifugation at 300 × g for 3 min and then washed with phosphate buffered saline (PBS). The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ±15 nm emission filter and the FL2-H channel 585 ±15 nm. For each analysis 10,000 events were recorded and analyzed using C6 Accuri software (BD Biosciences). The results obtained were verified by running duplicates and repeating experiments in two independent experiments.

p-AMPK/AMPK Ratio Western Blot Analysis. HepG2 cells were seeded at a density of 500,000 cells per/ mL (5 mL) in T-25 flask and incubated at 37 °C overnight in a humidified atmosphere containing 5% CO_2 in air. On the following day cultures were treated with the test compounds and incubated for an additional 24 h. Cells were detached and harvested by centrifugation, washed with saline buffer and the pellets were then lysed using 1X RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with $NaVO_4$, and protease inhibitor cocktail (Roche Applied Science), phosphatase inhibitor cocktail (PhoSTOP, Roche) and 1 mM phenylmethylsulfonyl fluoride (Sigma), and incubated on ice for 30 min with periodic mixing and two flash freeze cycles. These samples were cleared of insoluble material by centrifugation at 15,000 × g for 10 min at 4 °C. Total protein concentration was measured using a BCA kit (Thermo Fisher Scientific). NuPAGE LDS Sample Buffer (4X) and NuPAGE Reducing Agent (10X) was added to the lysate which was denatured at 70 °C for 5 min, then equal amounts of lysates (20 µg) were used for immunoblotting. Samples were resolved on a 4%–12% SDS–polyacrylamide Bis–Tris gels (Invitrogen) according to the manufacturer's recommendations and then proteins were transferred to PVDF membranes. After blocking with Superblock-TBS (Thermo Fisher Scientific) for 1 hour at room temperature, the primary antibodies against AMPKα phosphorylated at Thr172 (#2535), AMPKα (#2603), (all from Cell Signaling Technology, Beverly, MA, USA); β-actin (sc08432), (Santa Cruz Biotechnology, Dallas, TX, USA) were diluted with the corresponding primary anti-blocking solution and incubated at 4°C overnight with the membranes. Following three 10-min washes with Tris-buffered saline (TBST), the blots were incubated with horseradish peroxidase-linked secondary antibody IgGs (Cell Signaling Technology) at room temperature for 1 h. The blots were washed three times for 10 min with TBST, rinsed with deionized H_2O, and developed with enhanced chemiluminescence (ECL) (Azure Biosystem) using West Pico Chemiluminescent Substrate (Pierce Biotechnology). The Western blotting results were analyzed by grayscale scanning using AzureSpot software.